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1. Your reference — NSM P1B 40878

2. Patent application number *(The Patent Office will fill in this part)* — **9919713.9**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Queen Mary and Westfield College
University of London
Mile End Road,
London,
E1 4NS.

Patents ADP number *(if you know it)* 05573266001

If the applicant is a corporate body, give the country/state of incorporation — United Kingdom

4. Title of the invention — New Medical Use of High Density Lipoprotein

5. Full name, address and postcode in the United Kingdom to which all correspondence relating to this form and translation should be sent

Reddie & Grose
16 Theobalds Road
LONDON
WC1X 8PL

Patents ADP number *(if you know it)* 91001

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Country	Priority application *(If you know it)*	Date of filing *(day/month/year)*

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Number of earlier application	Date of filing *(day/month/year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

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Description	12
Claim(s)	0
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Priority documents	0
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11. I/We request the grant of a patent on the basis of this application.

Signature  Date 19 August 1999

12. Name and daytime telephone number of person to contact in the United Kingdom

P I BATES - Reddie & Grose
0171-242 0901

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NEW MEDICAL USE OF HIGH DENSITY LIPOPROTEIN

The present invention relates to the manufacture of medicaments for protecting against organ damage following ischaemia-reperfusion injury, using high-density lipoproteins HDLs and derivatives thereof. In particular, it relates to manufacture of medicaments for treatment and prevention of end-stage organ failure in haemorrhagic shock, and for treatment and prevention of tissue injury following myocardial infarction.

Many victims of sudden physical injury (for example, traffic accident victims) die because of end-stage organ failure. In patients with this condition, biochemical and biological changes (such as haemodynamic changes and microthrombus formation) occur in the blood and organs (such as liver and kidneys) due to shock and blood loss; this is a different action to "endotoxin" shock which arises due to bacterial infection. If end-stage organ failure is not halted or prevented, it will lead to permanent organ damage and death of the patient. There is a need for a pharmaceutical agent which can be administered as soon as possible after the physical injury, preferably at the site of the accident in order to prevent end stage organ failure, and which can also be used subsequently while transporting the victim from the accident site to casualty/hospital, and while the physical wounds are being treated.

High-density lipoproteins (HDLs) form a range of lipoprotein particles found in normal serum. Mature HDL particles are present in the form of a globular structure containing proteins and lipids. Within the outer layer of these particles are the more polar lipids, phospholipids and free

cholesterol, all having charged groups pointing outwards towards the aqueous environment. The more hydrophobic lipids, such as esterified cholesterol and triglycerides, reside in the core of the particle. Newly formed, or nascent, HDL particles lack the lipid core and are discoidal in shape. Protein components are embedded in the outer layer. The main protein component is apolipoprotein A-I (apo A-I), with smaller amounts of apo A-II, apo A-IV, apo CIII, apo D, apo E and apo J. Various other proteins reside on the HDL particle, such as lecithin-cholesterol acetyl transferase, PAF acetylhydrolase and paraoxonase.

The binding of activated leukocytes to the endothelium is the earliest observable cellular event in a number of acute and chronic inflammatory diseases. This binding is mediated by the expression of adhesion molecules on the surface of the endothelial cells which bind to corresponding molecules of similar function on leukocytes. Recently we have shown that pre-treatment of endothelial cells, *in vitro*, with HDL was able to inhibit the cytokine-induced expression of these adhesion molecules (Cockerill GW, Rye K-A, Gamble JR, Vadas MA, Barter PJ. Arterioscler Thromb. Vasc. Biol. 1995, 15: 1987-1994 1995, Cockerill GW Reed S.Int.Rev.Cytol: A survey of cell biology 1999). In addition, we have recently shown that HDL can inhibit cytokine-induced adhesion molecule expression in an acute inflammatory model in the pig (Cockerill et al., submitted 1999). The antiflammatory effects of HDL have thus been demonstrated in these models where cells/animals are pre-treated with lipoprotein.

End-stage organ failure following haemorrhagic shock results from the adhesion of polymorphonuclear leukocytes (PMNs) to the endothelium following their activation caused by

ischaemia and reperfusion injury. We have now found that administration of HDL or derivatives thereof prevents end-stage organ failure following ischaemia and reperfusion injury.

According to the present invention high density lipoprotein and or a derivative thereof is used in the manufacture of a medicament for the prevention or treatment of organ dysfunction following ischaemia and reperfusion injury.

Preferably, the medicament is for the treatment of end-stage organ injury or failure.

We have shown that, following ischaemia perfusion industry, HDL is able to perturb the damaging effects when given after the initial haemorrhagic shock has occurred. Our work suggests that at physiological levels (both *in vitro* and *in vivo*), native HDL particles are active in inhibiting the expression of adhesion proteins on endothelial cells. Prevention of expression of adhesion proteins on endothelial cells prevents binding of PMNs to the endothlium; thus administration of HDL prevents end-stage organ failure.

The high density lipoprotein may be the component of HDL that inhibits adhesion to the endothelial cells and subsequent activation of leukocytes or a derivative, molecule, homologue, or mimic thereof.

The inhibiting effect is not only present in venous endothelial cells but also on arterial endothelial cells and is independent of the nature of the lipid present in the HDL particles. Two effector molecules mediate the inhibitory effect namely apolipoprotein A-I (apo A-I and

apolipoprotein A-II (apo A-II) (Brouillette C.G. and Anatharamaiah G.M. Biochem.Biophys. Acta. 1256: 103-129. 1995; Massey J.B., Pownall H.J. Biochem.Biophys Acta. 999 : 111-120. 198); these two molecules have different efficacy of inhibition.

Preferably, the high density lipoprotein or derivative thereof is a peptide or protein derivative of the sequence of apo A-I or apo A-II, or a peptide or protein derivative functionally homologous to the active portions of apo A-I or apo A-II.

Preferably, the high density lipoprotein is reconstituted HDL.

The medicament may be administered to a patient in any conventional manner. Preferably the medicament is administered intravenously. Preferably, the medicament is administered using saline as a vehicle.

Preferably the medicament is provided in a portable dispenser, for example, for use at the site of an accident.

According to the invention in another aspect there is provided a method of treatment of organ dysfunction following ischaemia and/or reperfusion injury in a human patient which comprises the step of administering to a patient reconstituted high density lipoprotein and/or a derivative thereof in pharmaceutically acceptable form.

The present invention will now be illustrated with reference to the attached drawings in which :

FIGURE 1 shows alterations in mean arterial blood pressure (MAP) in rats subjected to (i) the surgical procedure without causing a haemorrhage and treated with vehicle for HDL (SHAM, open circle, saline, 3mg/kg i.v. bolus; n=9) or with HDL (SHAM + HDL black circle, 80mg/kg i.v. bolus injection, n=9) or (ii) haemorrhage for 1.5 h and upon resuscitation with the shed blood, control rats were treated with the vehicle (HS open squares, saline 3ml/kg i.v. bolus; n=10) or HDL (HS + HDL filled squares, 80mg/kg i.v. bolus injection, n=9);

FIGURE 1A shows a Table of heart rate in beats per minute(bpm) in all experimental groups studied before the haemorhhage -1.5 h and 1, 2, 3 and 4 h after resuscitation, Group 1: Rats were subjected to the surgical procedure without causing a haemorrhage and treated with a vehicle for HDL (saline, 3mg/kg i.v. bolus; n=9); Group 2: Rats were subjected to the same surgical procedure as Group 1 but treated with HDL (80mg/kg i.v. bolus injection; n=9)); Group 3: Rats were subjected to a haemorrhage for 1.5 h and upon reuscitation with the shed blood, control rats were treated with the vehicle (saline 1ml/kg i.v. bolus followed by an infusion of 1.5 ml/kg/h i.v., n=10); Group 4: rats were subjected to the same procedure as group 3 but treated with HDL (80mg/kg i.v. bolus injection; n=9).

FIGURE 2 shows plasma levels of (A) urea, (B) creatine, (C) AST, (D) ALT, (E) creatinine kinase (CK) and (F) lipase in rats subjected to the surgical procedure and experiment 2 described below;

FIGURE 3 shows a graph of mean fluorescence intensity (dependent on inhibition of E-selectin), as described below;

FIGURE 4(a) shows a graph of mean fluorescence intensity of HUVEC (veinous EC) against concentration of lipoproteins apo A-I and apo A-II for experiment 3, below; and

FIGURE 4(b) shows a graph of mean fluorescence intensity of HuAEC (arterial EC) against concentration of lipoprotein apo A-I and apo A-II for experiment 3, below.

As a demonstration of an embodiment of the invention, Experiment 1 describes the effects of human high-density lipoprotein (HDL) on the circulatory failure and multiple organ dysfunction injury (MODS) such as renal dysfunction and liver dysfunction caused by severe haemorrhage and resuscitation in the anesthetised rat. It should be noted that this is a model of end stage organ failure generated by ischaemia and reperfusion injury, and is not known to be a result of endotoxin release.

All experiments described herein were performed in adherence to the National Institute of Health guidelines on the use of experimental animals and in adherence to *Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986,* published by HMSO, London.

Experiment 1

The study was carried out on 26 Wistar rats (Tuck, Rayleigh, Essex, UK) weighing 250mg - 320g receiving a standard diet and water *ad libitum*. All animals were anaesthetised with thiopentone (120mg/kg i.p.) and anaesthesia was maintained

by supplementary injections of thiopentone as required. The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket. The right femoral artery was catheterised and connected to a pressure transducer (Senso-Nor 840, Senso-Nor, Horten, Noway) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR). These were displayed on a data acquisition system (MacLab 8e, AD Instruments, Hasting, UK) installed on an Apple Macintosh computer. The right carotid artery was cannulated to bleed the animals (see hereafter). The jugular vein was cannulated for the administration of drugs. The bladder was also cannulated to facilitate urine flow and to prevent the possibility of development of post-renal failure. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilise for 15 mins. Then, blood was withdrawn from the catheter placed in the carotid artery in order to achieve a fall in MAP to 50mmHg within 10 mins. Thereafter, MAP was maintained at 50mmHg for a total period of 90 mins by either withdrawal (during the compensation period) or re-injection of blood. It should be noted that in these experiments, the amount of shed blood re-injected during the 90 min period of haemorrhage did not exceed 10% of the total amount of the blood withdrawn. The amount of blood withdrawn for rats subjected to haemorrhage and treated with vehicle (control group) was 7.0 ± 0.4ml (SD); the amount of blood withdrawn from rats subjected to haemorrhage and treated with HDL (treatment group) was 7.0 ± 0.3ml ($p>0.05$). At 90 min after initiation of haemorrhage, the shed blood and an equivalent volume of Ringer lactic solution was re-injected into the animal.

The results are shown in Figures 1, 1A (Table 1) and 2.

FIGURE 2 shows plasma levels of (A) urea, (B) creatinine, (C) AST, (D) ALT, (E) creatinine kinase (CK) and (F) lipase in rats subject to (i) the surgical procedure without causing a haemorrhage and treated with vehicle for HDL (sham+saline,saline, 3ml/kg i.v. bolus i.v.; n=9) or with HDL (sham +HDL; 80mg/kg i.v. bolus injection, n=9, or (ii) haemorrage for 1.5.h and upon resuscitation with the shed blood, control rats were treated with the vehicle (hs (+ saline), saline 3ml/kg i.v. bolus n=9) or HDL (hs + HDL, 80mg/kg i.v. bolus injection, n=9). Haemorrhage and resuscitation resulted in significant increases in the serum levels of urea and creatinine (n=9), as demonstrated by the increase in urea and creatine concentration between "sham" and hs (control). This renal dysfunction was attenuated by the administration (5 mins prior to resuscitation) of HDL (80mg/kg. i.v., n=9; p,0.05; ANOVA followed by Bonferoni's test for multiple comparisons), as demonstrated by the concentration of urea and creatinine for "hs + HDL". Similarly, HDL attenuated the liver injury (as monitored by a rise in serum AST and ALT) - (C) and (D) - and the pancreatic injury (as measured by a rise in serum lipase - (F)) caused by haemorrhage and resuscitation. In contrast, HDL did not affect the delayed circulatory failure associated with haemorrhage and resuscitation (see Fig 1 and Fig.1A (Table 1)). Administration of HDL to rats, which were not subjected to haemorrhage, did not result in the alterations in the serum levels of urea, creatinine, AST, ALT or lipase (n=4) and, hence, was not toxic and the dose used.

In conclusion, administration of HDL attenuates the renal, liver and pancreatic dysfunction associated with ischaemia and reperfusion injury following haemorrhagic shock.

Experiment 2

This experiment demonstrates which components of the effective therapeutic agent HDL are responsible for protection against ischaemia-reperfusion injury; in this experiment, the ability of native HDL to inhibit cytokine-induced adhesion molecule expression on endothelial cells is compared with the ability of lipid-free apo A-I protein or protein-free lipid vesicles.

Cell culture : Human umbilical vein-derived endothelial cells (HUVEC) and human umbilical-derived artery endothelial cells (HUAEC) (Cockerill G.W, Meyer G, Noack L.Vadas MA, Gamble J.R. Lab.Invest.71 : 497-509.1994) were grown on gelatin-coated tissue culture flasks (Costar, High Wycombe, Bucks, UK) in medium 199 with Earle's salts (Gibco, Paisley, Scotland) supplemented with 20% foetal calf serum (FCS) (Gibco, Australia), 20mM HEPES, 2mM glutamine, 1mM sodium pyruvate, non-essential amino acids, penicillin and streptomycin, 50µg/ml endothelial cell growth supplement (Sigma, Dorset, UK) and 50µg/ml heparin (normal growth medium).

Flow cytometry : Cells were plated at 1 x 10^5 cells/30 mm well and incubated overnight at 37°C in 5% CO_2. Confluent monolayer cultures were then incubated (at concentrations indicated) for 19 hours with either, phosphate buffered saline (PBS) (vehicle control), native HDL, free apo A-I, phospholipid vesicles or discoidal HDL prepared with only apo A-I or apo A-II. Following these treatments the cells were washed gently in complete medium and TNFα (Miles Scientific) was added at 10ng/ml. Cells were then stained at 4 hours post stimulation in the following manner. Cells were washed in serum free medium and 200µl anti-E-selectin

(1,2B6) was added for 1 hour at 37°C. Cells were then washed in phosphate buffered saline (PBS) containing 5% newborn calf serum, 0.02% sodium azide, and 200µl of fluorescein isothiocyanate-conjugated secondary antibody added for 1 hour at 37°C. Cells were then washed three times in PBS and trypsinised, then centrifuged to form a pellet. The pellet was then resuspended in 2.5% formaldehyde in PBS containing 2% glucose and 0.02% azide and analyzed in a Coulter Epics Profile II flow cytometer.

Figure 3 shows that neither free apo A-I nor unilamellar vesicles (SUV) were able to inhibit TNFα-induced expression of E-selectin. This suggests that Apo A-I, the most abundant apolipoprotein in HDL, must be in a lipid particle in order to mediate inhibition of cytokine-induced adhesion molecule expression in endothelial cells. Both umbilical-derived venous (HUVEC) and arterial (HUAEC) endothelial cells were able to support the dose-dependent inhibition of cytokine-induced E-selectin expression by HDL (as shown by the decrease in intensity with increase of apo AI HDL from 0.25 t0 1.0mg/ml).

The therapeutic action of HDL is afforded by the apolipoprotein presented in a lipid particle, and cannot be mimicked by the whole protein alone, or lipid alone.

Experiment 3

To determine the efficacy of reconstituted discoidal HDLs particles containing either of the most abundant apolipoproteins (apo A-I or apo A-II), a comparison of the ability of these particles to inhibit cytokine-induced

adhesion molecule expression in HUVEC and HUAEC was carried out.

Cell culture : Human umbilical vein-derived endothelial cells HUVEC and human umbilical-derived artery endothelial cells HUAEC (Cockerill et al., 1995) were grown on gelatin-coated tissue culture flasks (Costar, High Wycombe, Bucks, UK) in medium 199 with Earle's salts (Gibco, Paisley, Scotland), supplemented with 20% foetal calf serum (FCS) (Gibco, Australia), 20 mM HEPES, 2mM glutamine, 1mM sodium pyruvate, nonessential amino acids, penicillin and streptomycin, 50µg/ml endothelial cell growth supplement (Sigma, Dorset, UK) and 50µg/ml heparin (normal growth medium).

Flow cytometry : Cells were plated at 1 x 10^5 cells/30 mm well and incubated overnight at 37°C in 5% CO_2. Confluent monolayer cultures were then incubated (at concentrations indicated) for 19 hours with either reconstituted discoidal HDL prepared with only apo A-I or apo A-II. Following these treatments the cells were washed gently in complete medium and TNFα (Miles Scientific) was added at 10ng/ml. Cells were then stained at 4 hours post stimulation in the following manner. Cells were washed in serum free medium and 200µl anti-E-selectin (1.2B6) was added for 1 hour at 37°C. Cells were then washed in phosphate buffered saline (PBS) containing 5% newborn calf serum, 0.02% sodium azide, and 200µl of fluorescein isothiocyanate-conjugated secondary antibody added for 1 hour at 37°C. Cells were then washed three time in PBS and trypsinised. The pellet was then resuspended in 2.5% formaldehyde in PBS containing 2% glucose and 0.02% azide and analyzed in a Coulter Epics Profile II flow cytometer.

Preparation of Reconstituted HDL Particles : Discoidal reconstituted A-I HDLs were prepared by the cholate dialysis method from egg yolk phosphatidylcholine, unesterified cholesterol, and apo A-I/apo A-II (Matz CE, Jonas A. Micellar complexes of human apolipoprotein A-I with phosphatidycholines and cholesterol prepared from cholate-lipid dispersion. *J.Biol.Chem.1982; 257; 4535-4540*). Egg yolk phosphatidycholine, unesterified cholesterol and sodium cholate were obtained from Sigma and used without further purification. Particle size was measured by nondenaturing gradient gel electrophoresis, and concentration of apo A-I and apo A-II was measured immunoturbidimetrically.

Results : Discoidal reconstituted HDL particles containing either apo A-I (open squares) or apo A-II (closed squares), as the sole protein, were able to inhibit TNFα-induced expression of both arterial and venous endothelial cells VCAM-1. Figure 4 shows reconstituted HDL containing apo A-I, as the sole proteins, having a t1/2 max of approximately 3 μMolar, whilst reconstituted HDL containing apo A-II as the sole protein has a give five-fold greater t1/2 max of 15 μMolar.

Conclusion : The therapeutic action of HDL can be mimicked using either apo A-I or apo A-II in reconstituted lipoprotein particle.

Figure 1

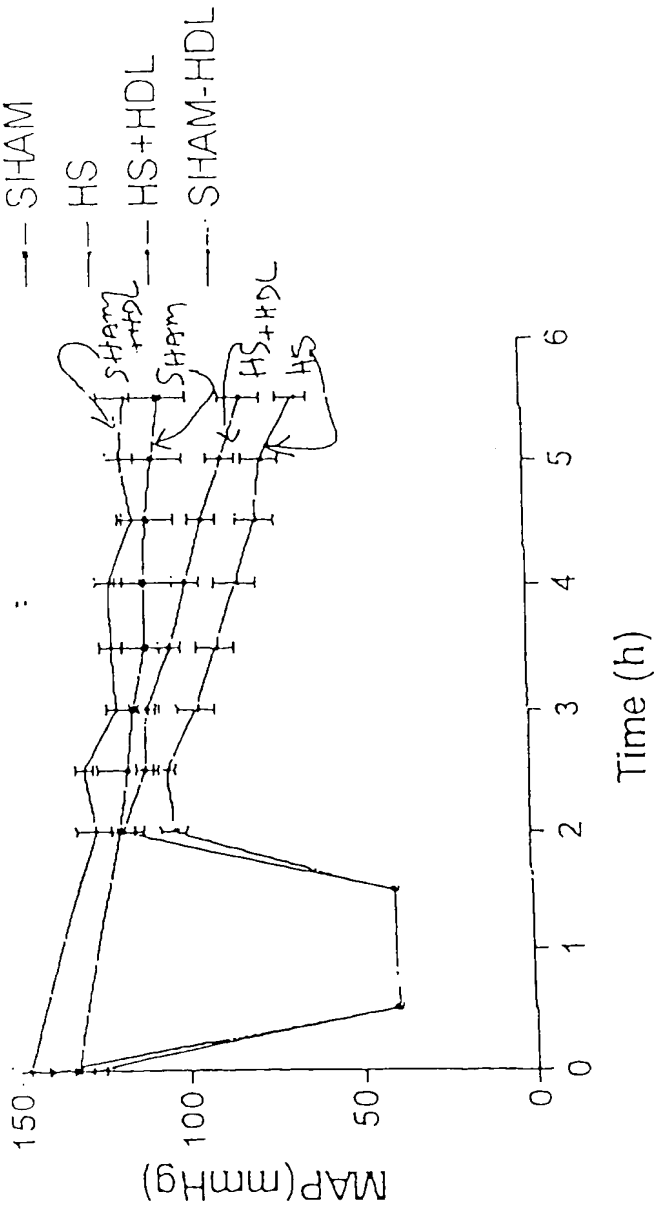

Figure 1A

(Table 1)

Group	-1.5 h	1 h	2 h	3 h	4 h
1. sham + saline	344±12	350±9	364±10	360±14	380±17
2. sham + HDL	380±14	397±12	399±7	396±9	384±16
3. HS + saline	368±15	397±10	380±18	365±16	345±23
4. HS + HDL	352±9	362±13	380±13	381±9	382±11

Figure 2A

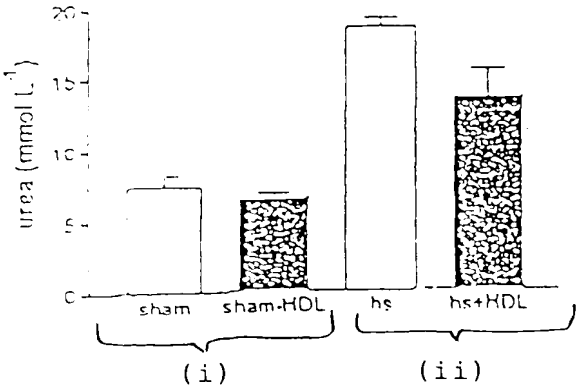

Figure 2B

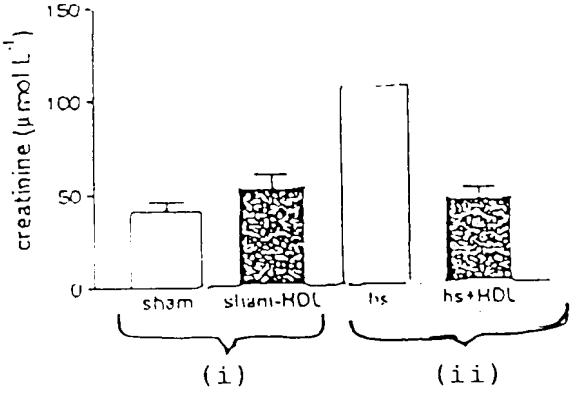

Figure 2C

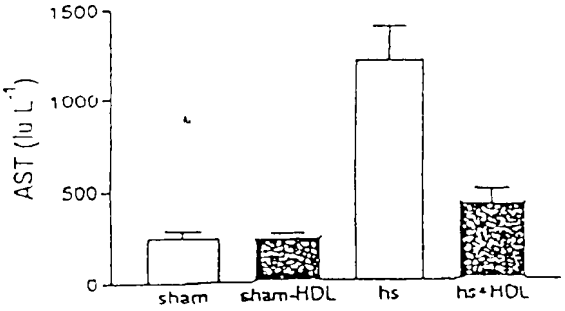

Figure 2D

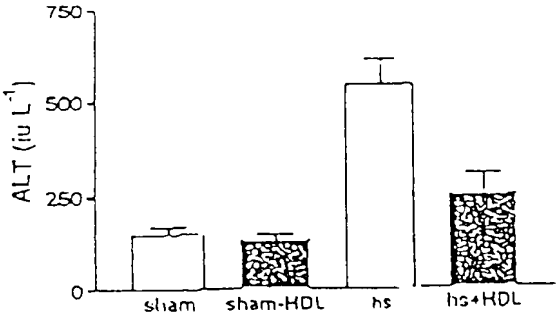

Figure 2E

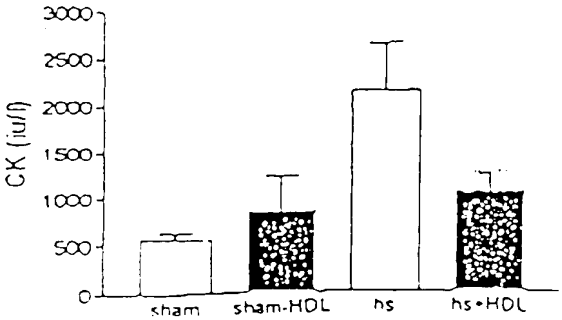

Figure 2F

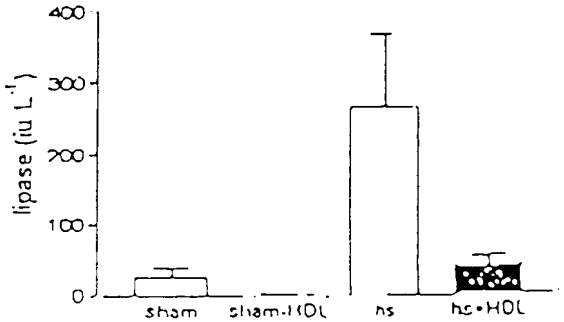

Figure 3

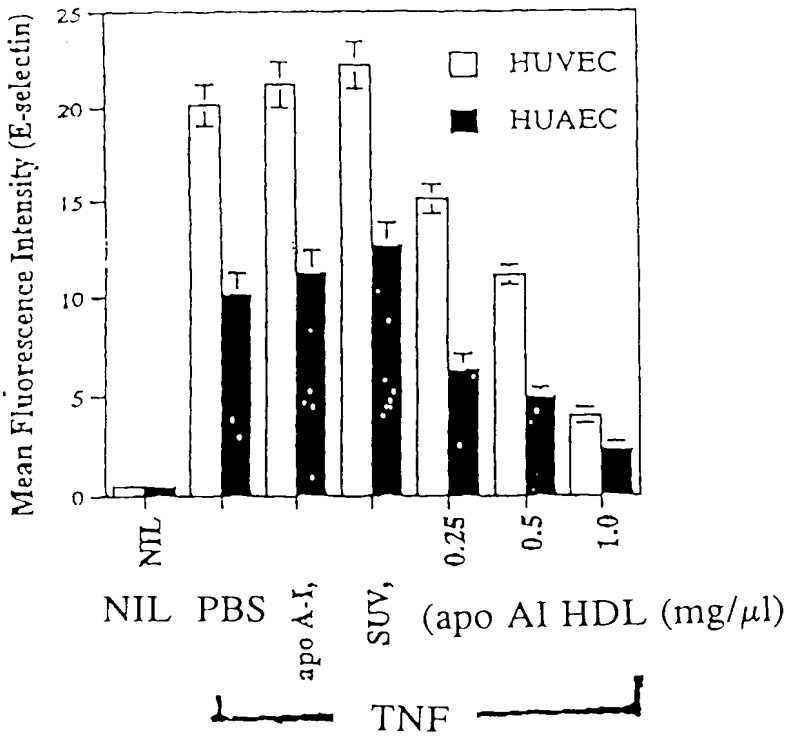
Mean Fluorescence Intensity (E-selectin)
25
20
15
10
5
0
HUVEC
HUAEC
NIL
0.25
0.5
1.0
NIL PBS apo A-I, SUV, (apo AI HDL (mg/μl)
TNF

Figure 4A

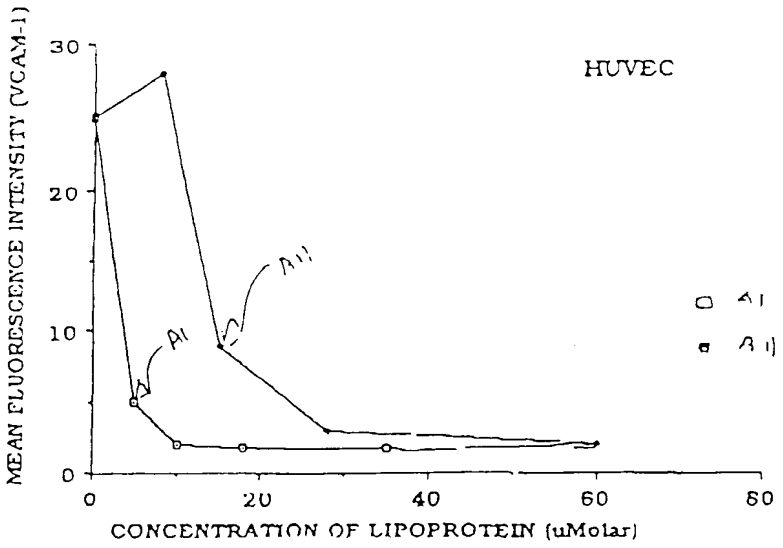

Figure 4B